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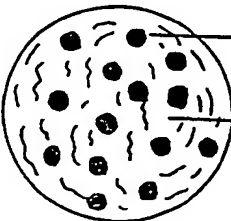
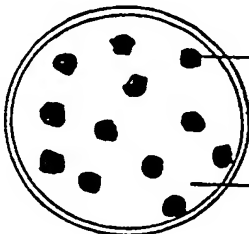
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US90/04381</p> <p>(22) International Filing Date: 6 August 1990 (06.08.90)</p> <p>(30) Priority data: 389,455 7 August 1989 (07.08.89) US</p> <p>(71)(72) Applicants and Inventors: SCHLAMEUS, Herman, Wade [US/US]; 7106 Quail Garden, San Antonio, TX 78250 (US). FOX, William, Casey [US/US]; 7418 Pebblewood, San Antonio, TX 78250 (US). MANGOLD, Donald, Jacob [US/US]; 14711 Bold Venture, San Antonio, TX 78248 (US). TRIPLETT, Robert, Gill [US/US]; 2906 Bee Cave, San Antonio, TX 78231 (US). HOLT, George, Richard [US/US]; 100 Pin Oak Forest, San Antonio, TX 78232 (US). AUFDEMORTE, Thomas, Bruce [US/US]; 1638 Vista Del Monte, San Antonio, TX 78219 (US).</p>		<p>(74) Agent: GOODMAN, Rosanne; Fulbright &amp; Jaworski, 300 Convent Street, Suite 2200, San Antonio, TX 78205 (US).</p> <p>(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: COMPOSITION AND METHOD OF PROMOTING HARD TISSUE HEALING</p> <p>(57) Abstract</p> <p>Osteoprogenitor cells encapsulated in alginate and alternatively, additionally encapsulated in polylysine and/or agarose promote regeneration of bone at the site of implantation. The present invention provides a composition comprising osteoprogenitor cells embedded or encapsulated in alginate and the use of said microcapsules for the facilitation of bone regeneration.</p> <div style="display: flex; justify-content: space-around; align-items: center;">  <div style="text-align: right;"> <p>Live Cells</p> <p>Algin Matrix</p> </div> </div> <div style="display: flex; justify-content: space-around; align-items: center; margin-top: 20px;">  <div style="text-align: right;"> <p>Live Cells</p> <p>Polylysine-Algin Membrane</p> <p>Liquid Medium</p> </div> </div> <p style="text-align: center; margin-top: 20px;"><b>TWO TYPES OF ARTIFICIAL CELLS</b></p>		

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COMPOSITION AND METHOD OF PROMOTING  
HARD TISSUE HEALING

15 Field of the Invention

The present invention relates generally to the field of hard tissue healing and, more particularly, to the field of biodegradable implantable microcapsules to stimulate the natural process of hard tissue regeneration and bone wound healing.

Background of the Invention

Defects in bone or osseous structures will initiate the process of bone healing. Healing often involves the replacement of injured tissue by connective tissue and leaves a scar. Bone, under optimal conditions, heals by regeneration in which injured tissues are replaced by their own kind and leave no scar. The success of regeneration following injury depends, among other things, on the type of injury, the adequacy of treatment and the systemic health of the patient. Osseous repair involves at least six physiological stages: impact, induction, inflammation, soft callus formation, hard

35

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1 callus formation, remodeling and regeneration.  
Heppenstall, Fracture Treatment and Healing, W.B.  
Saunders, Philadelphia, 1980, page 35.

5 With inadequate treatment, severe injury and/or  
metabolic bone disease, fracture healing is significantly  
retarded. For example, in the case of a metabolic bone  
disease such as osteoporosis, 40% of patients with  
decreased bone mass due to osteoporosis showed a markedly  
impaired fracture repair rate. Only 33% of women in whom  
10 significant osteoporosis was present were able to achieve  
a solid union following femoral neck fractures. In  
comparison, in 90% of women with physiologically normal  
bone mass a successful union was achieved. Lane et al.,  
Osteoporosis, Orthopedics clinics North America 15: 711  
15 (1984); Arnold, J. Bone Joint Surg. 66A: 847 (1984);  
Scileppe et al., Surg. Form 32: 543 (1981).

It is estimated that there are 200,000 hip  
fractures in osteoporetic women in the United States  
annually with a 40% mortality rate due to complications of  
20 repair of these fractures. As a result, there is a  
significant need to facilitate fracture repair in these  
types of patients. In addition, fractures in young  
accident and trauma victims result in loss of numerous  
productive days from the work place. For example, it  
25 takes an average of six weeks to complete repair even  
simple bone injuries in healthy individuals.

Bone fractures and bone wound healing following  
trauma or surgery account for considerable morbidity and  
mortality. For example, femoral neck fractures in  
30 patients under forty may be associated with avascular  
necrosis in as many as 40% of cases complicated by  
non-union. Kyle et al., Young Femoral Neck Fractures,  
Presented at the 52st Annual Meeting of American Acadeym  
of Orthopedic Surgery, Atlanta, Ga. (1984). Many other  
35 examples could be cited of the need for more expeditious

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1 methods to facilitate and/or accelerated fracture or hard  
tissue defect repair.

In addition, recent technological advances have  
made the replacement of joints and defective or diseased  
5 hard tissues common surgical procedures.

Since the feasibility of the preparation of  
artificial cells was first demonstrated in 1957 by Chang  
(Chang, T.M.S. (1964) Science:146, 524), numerous  
approaches to their production and use have been  
10 evaluated. Artificial cell membranes have been reported  
using a variety of synthetic and biological materials to  
give the desired membrane properties. A large variety of  
materials can be enclosed (microencapsulated) in  
artificial cells. This includes single and multienzyme  
15 systems, cell extracts, and combined enzyme-adsorbent  
systems (Chang, U.S. Patent No. 4,642,120). Biological  
cells have been encapsulated to prevent them from being  
adversely affected by external factors and immunological  
rejection (Chang, Biomedical Applications of Immobilized  
20 Enzymes and Proteins (Plenum: New York, 1977) Vols. 1 and  
2; Mosbach et al. (1966) Acta Chem. Scan. 20: 2807; Lim et  
al. (1980) Science 210:908). More recently the  
microencapsulation of living biological cells that can be  
maintained in culture has been disclosed (Lim et al.  
25 (1980) Science 210: 908; U.S. Patent No. 4,391,909).

U.S. Patent No. 4,663,286 (Tsang et al.)  
discloses a process for encapsulating material is  
described for forming a capsule utilizing an alginate  
polymer with a polyvalent cation.

30 U.S. Patent No. 4,642,120 (Nevo et al.) discloses  
the repair of cartilage and bones by employment of a  
composition provided in gel form. The gel comprises  
certain types of cells. These may be committed embryonal  
chondrocytes or any type of mesenchymally-derived cells  
35 which may differentiate into chondrocytes, generally as a

1 consequence of the influence of chondrogenic inducing  
factors, in combination with fibrinogen, antiprotease,  
thrombin, and other factors. According to U.S. Patent No.  
4,642,120, the cells should be of the same species as that  
5 to which the composition is transplanted. Incorporation  
of extracellular matrix (ECM) of chondrocytes, other  
hormones and/or growth factors such as SM (Somatomedin or  
IGF-I), FGF (fibroblast growth factor), CGF (cartilage  
growth factor), BDGF (bone derived growth factor) or a  
10 combination of any of these in the gel is also disclosed.

U.S. Patent No. 4,472,840 (Jefferies) discloses a  
method of inducing osseous formation by implanting bone  
graft material. Both demineralized bone particles (DBP)  
and bone inductive protein have demonstrated the capacity  
15 to induce the formation of osseous tissue in animal and  
human experiments. Reconstituted collagen conjugate is  
known to be highly biocompatible and can be fabricated in  
a variety of configurations, especially as a sponge. This  
material can be used as a grafting implant in plastic and  
20 reconstructive surgery, periodontal bone grafting, and in  
endodontic procedures. Structural durability is enhanced  
by crosslinking with glutaraldehyde which is also used to  
sterilize and disinfect the collagen conjugate prior to  
implantation.

25 U.S. Patent No. 4,132,746 (Urry et al.) discloses  
a crosslinked insoluble polypentapeptide elastomer capable  
of calcification by withdrawing calcium ions from a serum  
medium, thus making it useful as a calcifiable matrix for  
the formation of an artificial bone structure. The  
30 calcifiable material can be treated to make it useful in  
artificial vascular wall formation.

U.S. Patent No. 4,609,551 (Caplan et al.)  
discloses a material for stimulating growth of cartilage  
and bony tissue at anatomical sites. The material  
35 consists of a composition with a fibrin or allograft

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1 matrix containing soluble bone protein and fibroblast  
cells.

Summary of the Invention

5 In order to facilitate the healing of bone and  
other hard tissue fractures and defects and facilitate  
structural implant fixation, a microcapsule has been  
developed. Specifically, it has been discovered that  
osteoprogenitor cells can be embedded in or encapsulated  
10 by biocompatible materials and nonetheless retain their  
viability and biological function. The biocompatible  
encapsulating materials useful in practicing this  
invention can have different rates of biodegradability.  
The biocompatible material may be readily biodegradable,  
slowly biodegradable or relatively resistant to  
15 degradation in biological fluids. A readily  
biodegradable material is one that is degraded 50% or  
more within hours to several days by contact with  
biological fluids. A slowly biodegradable material will  
degrade at least 50% when in contact with biological  
20 fluids for more than several days up to a week or several  
weeks. A material resistant to biodegradation is one  
which retains its integrity for at least several weeks in  
the presence of biological fluids.

Materials which are readily biodegradable  
25 (bioerodable) include naturally-occurring polymers such as  
alginates, polylysine, cellulose polymers, e.g.,  
methylcellulose, collagen, gellan gum, casein, chitosan,  
and the like. Materials which are slowly degradable  
include some polyesters, and polyanhydrides.  
30 Biocompatible materials which are relatively resistant to  
biodegradation include titanium oxide, hydroxyapatite,  
biocompatible metal compositions, biocompatible ceramic  
compositions, and the like. The microcapsules of the  
present invention can comprise one biodegradable material  
35 or a combination of two or more biodegradable materials.

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1 In the latter case the microcapsule may contain  
biocompatible materials of varying rates of  
biodegradability.

5 A microcapsule comprising one or more  
biodegradable materials can itself be coated or further  
encapsulated by a less readily degradable substance in  
order to further delay complete release of the  
encapsulated material. By carefully choosing the  
10 materials used as the initial encapsulating material and  
the subsequent coating or encapsulating material, one of  
skill in the art may control the rate of release of one or  
several encapsulated materials, including the encapsulated  
osteoprogenitor cells. For instance, in one embodiment,  
alginate alone can be used as the sole encapsulating  
15 material. In a second embodiment, biodegradability is  
retarded by coating thus-prepared alginate microcapsules  
with a polyanionic polymer such as polylysine.

In yet another embodiment a core material  
relatively resistant to biodegradation, such as a ceramic  
20 material to which another material, e.g., one of the above  
mentioned growth factors, has been bound and from which  
this other material is slowly released, e.g., from the  
surface of the core material, may be encapsulated within a  
more readily biodegradable material which itself contains  
25 the same or other treating materials, e.g., the same or  
another growth factor, antiviral agent, hormone, in order  
to sustain release of one or more of the encapsulated  
materials. For instance, a microcapsule comprising woven  
titanium mesh mixed with collagen may be also be embedded  
30 within the algin microcapsule containing osteoprogenitor  
cells. Prosthetic devices formed of the present invention  
will facilitate fixation of orthopedic devices or dental  
implants by enhancing the bone regeneration at the site of  
prosthetic implantation. In another embodiment, fixation  
35 of orthopedic implants at the surgical site can be

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1 facilitated by implantation of the composition of the  
present invention comprising ceramic hydroxyapatite  
adsorbed with bone derived growth factor or any other  
material which stimulates the differentiation or growth of  
5 osteoprogenitor or cartilage progenitor cells with the  
progenitor cells in the formable materials useful for  
practicing the present invention. This embodiment allows  
a stable and solid support for replacement and/or  
reconstruction of defective hard tissues while  
10 additionally providing the necessary progenitor cells to  
repair and/or replace the defective hard tissue  
structures.

As indicated above, microcapsules prepared in  
accordance with this invention can additionally contain  
15 materials which aid in bone healing or in the prevention  
or treatment of complications of trauma. Such additional  
materials can include, but are not limited to,  
extracellular matrix of chondrocytes (ECM), hormones,  
growth factors such as somatomedins, fibroblast growth  
20 factor, bone morphogenic protein, platelet derived growth  
factor, bone inductive growth factor, osteoinductive  
growth factor, cartilage derived growth factor,  
prostaglandins, macrophage derived growth factors, bone  
derived growth factor, skeletal derived growth factor,  
25 epidermal growth factor, transforming growth factor  $\beta$ ,  
growth factor, cytokines, and the like, or a combination  
of any of these. Such materials may alternatively be  
termed herein hard tissue promoting factors. Other agents  
which aid in treatment or prevention of the complications  
30 of trauma may additionally be included. Examples of such  
other agents are, without limitation, antiviral agents,  
antibacterial agents and the like. The above agents and  
factors may be used alone or in combination in practicing  
the present invention. Such materials can be prepared by  
35 any method known to those skilled in the art, including

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1 purification from naturally occurring sources and  
recombinant technology.

5 The microcapsules of this invention, coated or  
uncoated, e.g., with polylysine, can be further surrounded  
by a material that can be formed into a hydrogel wafer,  
such as agar, gelatin, gellan gum or the like, in order to  
facilitate handling and transfer or implantation of  
encapsulated material(s) into the site of treatment.

10 The present invention provides compositions and a  
method to facilitate the healing or regeneration of bone,  
for instance, at fracture sites. This method comprises  
implantation or injection of any of the compositions of  
the present invention into a site or a device in an  
individual at which bone fixation, reconstruction,  
15 regeneration or healing is desired. The osteoprogenitor  
cells then proliferate and cause the deposition of new  
bone material at the implantation or injection site.

The present invention also provides compositions  
and a method to facilitate the regeneration and healing of  
20 cartilagenous tissues.

It is, therefore, an object of the present  
invention to provide a composition to augment and/or  
facilitate the regeneration or healing of bone tissue at  
fracture sites.

25 A further object of the present invention is to  
provide a wafer delivery system for encapsulated  
osteoprogenitor cell-containing compositions of the  
present invention.

30 Yet another objective of the present invention is  
to provide viable encapsulated osteoprogenitor cells for  
implantation and timed-release at bone fracture sites to  
augment and facilitate healing of the fracture.

A still further object of the present invention  
is to provide a method of stimulating the healing of bone  
35 fractures.

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1           These and other objects, as well as the nature,  
scope, advantages and utilization of this invention, will  
become readily apparent to those skilled in the art from  
the following description, the drawings and the appended  
5       claims.

Brief Description of the Drawings

Figure 1 shows a schematic of the two capsule  
types.

10          Figure 2 shows a schematic of the type of  
apparatus used to form one type of microcapsule.

Detailed Description of the Invention

15          In order to accomplish the above objects and  
objectives, the present invention provides, in one  
embodiment, osteoprogenitor cells embedded or encapsulated  
in an alginate matrix.

20          In one embodiment of the present invention,  
osteoprogenitor cells have been encapsulated, viability  
maintained within artificial membranes, and the cells when  
implanted in an animal model, subsequently proliferate and  
maintain their capacity to induce osteogenesis.

25          The osteoprogenitor cells useful in carrying out  
the present invention can be any cells capable of inducing  
the formation of regenerated bone (or cartilage)(or the  
deposition of calcium?). Preferably, these cells are  
autologous bone progenitor cells harvested from the  
individual in need of such treatment. These cells may be  
harvested from the site of the injury or from a distant  
site for transplantation to the injury site. The primary  
cells may be used directly or may be expanded by passage  
30       in cell culture. In another embodiment, the  
osteoprogenitor cells may be harvested from another  
individual of the same specie as the individual to be  
treated. However, the cells may also be selected from  
the group consisting of cell lines derived from any  
35       mesenchymally cells which will differentiate to form

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1 osseous or cartilaginous tissue. In selecting the  
osteoprogenitor cells for use in the present invention it  
is only important to try to minimize as much as possible  
the rejection of the implanted cells for the period  
5 necessary to induce the regeneration of new bone.

The preferred composition of the present  
invention comprises osteoprogenitor cells embedded or  
encapsulated in a biodegradable material. The cells may  
either be embedded in a matrix material by being dispersed  
10 within the matrix material itself or by surrounding the  
cells with a biodegradable material. In either case, in  
order to decrease the rate of release of the  
osteoprogenitor cells from the microcapsule, the cells may  
be further encapsulated in a nonbiodegradable material or  
15 a material which has a prolonged integrity in the host  
such as polylysine. Preferably, the matrix material is an  
alginate, such as sodium alginate. The matrix material  
may also be selected from the group consisting of gellan  
gum, chitosan, or agarose.

20 The method of encapsulating the osteoprogenitor  
cells comprises embedding or encapsulating the cells in a  
biodegradable material by any of the techniques known to  
those of skill in the art. Preferably the osteoprogenitor  
cells are encapsulated by a modification of the method  
25 disclosed in U.S. Patent No. 4,391,909, incorporated  
herein by reference. Briefly, osteoprogenitor cells were  
gently dispersed in a solution of sterile sodium alginate  
and pumped through a needle into a collection bath of 1.3%  
calcium chloride containing Tween 20. The alginate  
30 embedded cells, also termed herein microcapsules, were  
harvested, washed with saline and either used directly for  
implantation or injection into the treatment site or  
further encapsulated to prolong the integrity in the host.

In a preferred embodiment, the microcapsules were  
35 formed into wafers to facilitate implantation. These

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1 wafers were preferably composed of agar, such a wafer is  
described in Example 4. The wafer can be of any material  
that is biocompatible and can be formed into a hydrogel  
5 having characteristics similar to agar such that the  
handling and placement of the microcapsules at the  
treatment site is facilitated.

The method of treating bone fractures of the  
present invention comprises implantation of the  
osteoprogenitor microcapsules of the present invention  
10 into a fracture site of an individual and allowing  
sufficient time for the formation of new bone at the  
treatment site. The osteoprogenitor microcapsules may be  
implanted by surgical procedures known to those of skill  
in the art or may be injected into the fracture site  
15 utilizing a suitable pharmaceutical carrier. The choice  
of such carriers will be obvious to those in the art.

The term "individual" is meant to include any  
animal, preferably a mammal, and most preferably a human,  
cat, dog, or horse.

20 Artificial cell preparation was carried out in a  
sterile environment. All equipment, materials, solutions,  
etc. were either sterilized by the appropriate means or  
purchased as sterile before use in the process.

Having now generally described the invention, a  
25 more complete understanding can be obtained by reference  
to the following specific examples. These examples are  
provided for purposes of illustration only and are not  
intended to be limiting unless otherwise specified.

#### 30 Example 1

##### PROCEDURE FOR ENCAPSULATION OF OSTEOPROGENITOR CELLS

##### A. Preparation of osteoprogenitor cells.

Cells were isolated from canine trabecular bone  
specimens. The specimens represented material obtained by  
35

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1 biopsy of the iliac crest of four research grade mongrel  
dogs numbered as follows: 4452, 4386, 4593, and 4467.  
The biopsy specimens from each dog were processed  
individually in order to permit autologous implantation of  
5 the cellular material at a later date, thereby  
circumventing any possible rejection response and  
eliminating the need for immune suppression of the host  
dogs.

The biopsy material was washed multiple times in  
10 Dulbecco's modified Eagle's medium (DMEM) containing  
penicillin (1000 units/ml), streptomycin (1000 ug/ml), and  
amphotericin-B (0.25 ug/ml) to remove adherent tissue and  
debris. The bony trabeculae were then cut into small  
pieces (1-2 mm<sup>2</sup>) followed by a second series of washings  
15 to remove hematogenous elements. The resulting clean  
pieces of bone were placed in a 100 mm cell culture dish  
in the absence of media and incubated at 37°C in an  
atmosphere of O<sub>2</sub>/CO<sub>2</sub> (95/5 v/v). After 20 minutes, 10  
ml DMEM containing 10% newborn calf serum (NCS) was  
20 carefully added to the dish without disturbing the bone  
fragments. The dishes were returned to the incubator and  
left undisturbed for 5 days. Subsequently, the media was  
changed every three days to fresh DMEM, 10% NCS. After 23  
days of culture, the cells which had migrated from the  
25 bone fragments onto the surface of the culture dish were  
removed with trypsin/EDTA (0.125%/1 mM).

These cells were placed in a T-75 culture flask  
and designated first passage cells. The cells were  
passaged two more times to yield third passage cells  
30 which, when confluent, were encapsulated as described  
below (Runs 1-30A through 1-31B). Examination of aliquots  
of the encapsulated cells suspended in DMEM containing the  
vital dye trypan blue, indicated that the cells had  
retained their viability during the encapsulation.

35

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1 procedure. The encapsulated cells were maintained in DMEM  
containing 10% NCS at 37°C in an atmosphere of  $O_2/CO_2$   
(95/5 v/v) for 24-48 hours prior to preparation for  
5 implantation into nonunion sites prepared in the radii of  
dogs. Viability experiments revealed that the  
encapsulated cells could be maintained in this manner for  
three days without a decrease in cell number. In fact,  
the cell number increased by 70-90% during this time  
period.

10 Cells for implantation in nonunion fracture sites  
in dogs were harvested and grown in culture as described  
above. Osteoprogenitor cells were incubated in an  
incubator (37°C) until ready for use in the encapsulation  
process.

#### 15 B. Encapsulation of Cells

Cells were encapsulated by a modification of the  
method described in U.S. Patent No. 4,391,909 (Lim). Two  
types of encapsulated cells were prepared. In one, cells  
were encapsulated (or embedded) in an algin matrix. In  
20 the second, the process was carried further and the  
alginate embedded cells were further encapsulated using  
poly-L-lysine/alginate as the capsule membrane. A schematic  
of the two capsule types is shown in Figure 1.

The encapsulated cells were prepared as follows  
25 and used for implantation into animals to demonstrate the  
effect on fracture healing. Cells from several flasks  
were combined, placed in a 15-ml sterile culture tube and  
rinsed 3 times with sterile 0.9% saline solution. After  
decanting the saline solution from culture tube, 10 ml of  
30 sterile sodium alginate solution (about 1%) was added.  
The alginate used for most of the cell encapsulation was  
sterile Macrocarrier\* solution obtained from Bellco Glass,  
Inc. The cells were gently dispersed and the  
cell/alginate solution was transferred to a sterile  
35 syringe. The syringe was placed in a sterile pump device

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1 and connected to the encapsulation device with sterile  
tubing.

5 A sterile collection bath (containing a 1.3%  
calcium chloride solution with 0.25 ml of 10% Tween 20)  
was placed under the encapsulation device. The cells were  
encapsulated in the alginate and collected in the  
collection bath. After the alginate encapsulated cells  
remained in the collection bath for 3-5 minutes, they were  
passed through fine wire screen baskets. The alginate  
10 embedded (encapsulated) cells were then rinsed 2 times  
with 0.9% saline solution and used in this form as the  
alginate matrix cell preparation.

In some situations when it is desirable to  
provide polylysine encapsulated osteoprogenitor cells, the  
15 alginate embedded (encapsulated) cells were rinsed once  
with a polylysine solution, preferably about 0.2%. The  
poly-L-lysine used in the encapsulation was obtained from  
Sigma Chemical Company and had a molecular weight of  
approximately 38,000. The cells were then incubated in  
20 the polylysine solution for 5-7 minutes, rinsed 2 times  
with 0.9% saline solution, and finally, rinsed once with  
an approximately 1.5% sodium citrate solution by  
incubating the encapsulated cells in the sodium citrate  
solution for 5-7 minutes. The cells were then rinsed 2  
25 times with 0.9% saline solution and 3 times with DME  
(Dulbecco's Modified Eagle's Medium) for 2-3 minutes.

The cells suspended in approximately 40 ml DME  
were transferred to a sterile T-75 flask and incubated at  
37°C until implantation.

30 The results of the encapsulation procedures are  
shown on Table 1. In the initial runs (1-1A through 1-9D)  
only placebo capsules were prepared in order to adjust  
process parameters to produce the desired type of  
capsule. Matrix materials evaluated during this period  
35 included alginates, casein, chondroitin sulfate, and

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1 collagen. In the preferred embodiment, spheres were  
formed using sodium alginate collected in a calcium  
chloride (CaCl<sub>2</sub>) bath as shown in Run 1-7A in Table 1. In  
5 forming the capsule, air regulation was used to control  
the droplet size. A schematic of the apparatus used is  
shown in Figure 2.

Runs following 1-12A were carried out with  
encapsulating live cells unless otherwise stated. The  
encapsulation of osteoprogenitor cells are designated as  
10 1-30A through 1-31B in Table 1.

Histopathologic analysis was performed on  
encapsulated cells maintained in vitro as well as on  
tissues removed at necropsy from animals implanted with  
microencapsulated osteoprogenitor cells for in vivo  
15 evaluation. This was accomplished in three phases as  
described in Examples 2, 3, and 4, below.

## Example 2

### In Vitro Cell Analysis

20 Following encapsulation of the cells, in vitro  
studies were conducted to determine osteoprogenitor cell  
viability and define their morphology within artificial  
cell membranes. Histologic sections were prepared and  
stained with hematoxylin and eosin using encapsulated  
25 cells in the following combinations:

- K-1 Alginate + U2OS cells (an osteosarcoma cell line)
- K-2 Polylysine + U2OS cells
- K-3 Alginate + normal dog cells (animal #4452)
- 30 K-4 Polylysine + normal dog cells (animal #4452)
- K-5 Alginate + FL cell tumor (a transformed human  
tumor cell line capable of bone formation)

U2OS cells encapsulated in alginate appeared as  
small nests or colonies numbering approximately 2-15  
35 cells, each with an average of approximately 10 cells per

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1 group. The cells had basophilic staining nuclei which  
were round and regular with prominent nucleoli noted at  
random. Cell cytoplasm was moderately eosinophilic and  
cell boundaries were relatively distinct. The algin  
5 matrix was amorphous and slightly basophilic but obviously  
degrading as a consequence of the histologic processing  
procedure necessary to produce the sections.

U2OS cells encapsulated in polylysine also  
appeared as clusters with morphology not significantly  
10 different from that described above, however, the  
artificial polylysine membranes were histologically  
distinct as slightly basophilic undulating cuticular  
surfaces enclosing cell nests. The undulation was  
interpreted as an artifact of dehydration, again necessary  
15 for processing.

Normal dog cells when encapsulated in alginate,  
appeared as isolated groups, usually of 2-3 cells.  
Morphologically the cells had the characteristics of  
osteoblasts with eccentrically located round nuclei and  
20 relatively conspicuous eosinophilic cytoplasm. In some  
cells there was evidence of a perinuclear eosinophilic  
condensation typical of osteoblasts. Again, the alginate  
membranes appeared to be degrading as a result of the  
histological preparation.

25 Normal osteoprogenitor dog cells encapsulated in  
polylysine showed similar morphology to those encapsulated  
in alginate alone. Again, polylysine membranes were  
distinct as described with the U2OS cells above.

Alginate embedded FL cells also showed isolated  
30 cells or groups of 2-4 cells with round, eccentrically  
located nuclei, occasional prominent nucleoli and  
eosinophilic cytoplasm, but differing from U2OS cell lines  
in that clusters were in general smaller and less numerous  
within the artificial membranes.

35

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1 In summary, all artificial cell preparations  
contained viable cells with morphology varying as to the  
derivation of the particular cell type indicating that no  
deleterious effects resulted from the encapsulation  
5 process.

Alginate cell membranes degraded during  
histological processing and thus were not visible in  
subsequent sections produced from animal studies.  
Polylysine membranes were more distinct and durable and  
10 remained visible at least in early phases of the animal  
studies. The interpretation of the in vivo data shown in  
the Examples below was made in accordance with these  
observations .

These studies demonstrate that cells may be  
15 encapsulated, their viability maintained, and sections  
prepared for histologic analysis. Intact cells were noted  
within the confines of the artificial membranes and, as a  
consequence, these formulations rendered viable cells for  
implantation studies.

20

### Example 3

#### In Vivo Studies of Encapsulated Cell Lines

##### Implanted in Nude Mice

Cell viability following encapsulation was  
25 evaluated in vivo using FL cells, a transformed line of  
human amnion cells capable of tumor formation in the nude  
mouse. The rationale for these experiments follows.  
Cells encapsulated in alginate and implanted beneath the  
skin of the nude mouse formed tumors as rapidly as  
30 nonencapsulated cells injected subcutaneously, since the  
alginate is rapidly dissolved in vivo. Formation of  
tumors by cells encapsulated in polylysine was delayed,  
since polylysine is not readily dissolved in the host and  
cells first have to multiply within the capsules in  
35 sufficient mass to burst them.

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1           Encapsulated FL cells (Runs 1-15B, 1-16B, 1-36A,  
and 1-36B) were maintained overnight at 37°C in an  
atmosphere of O<sub>2</sub>/CO<sub>2</sub>(95/5 v/v). The following morning 0.5  
5           ml alginate or polylysine encapsulated cells were  
surgically implanted beneath the skin of 3-week old nude  
mice of the nu/nu strain (Harlan). The mice were  
sacrificed at 16 and 32 days after implantation for gross  
and histological evaluation of tumor formation.

10           FL cell lines encapsulated in alginate and  
implanted for a period of 16 days, demonstrated at  
necropsy, viable cells with histologic features remarkably  
similar to those described in the in vitro experiments  
with the exceptions that the cell clusters were now much  
larger, often forming confluent nests in excess of several  
15           hundred cells.

          Alginate membranes, as expected, were not visible  
but the general outlines of the artificial cells were  
present in some areas, perhaps attributable to  
fibrocollagenous connective tissue proliferating in  
20           proximity with the artificial cell membranes. In other  
areas, FL cells had grown into confluent nests with the  
subcutaneous tissue and muscles, violating and disrupting  
the boundaries of the artificial cell membranes. In these  
areas of host tumor interface, conspicuous bone and  
25           osteoid production was noted.

          FL cells encapsulated in polylysine and implanted  
for 16 days again showed large viable cell clusters with  
morphologic features as described with the exception that  
the cell membranes of polylysine remained intact. Most  
30           cell groups within the membranes had grown to confluency.  
No evidence of cell penetration into adjacent tissues, as  
was noted above, was apparent. No bone or osteoid  
production was visible.

          FL cell lines encapsulated in alginate/polylysine  
35           harvested 32 days after implantation showed a large bulk

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1 of tumor (larger than 2.0 X 1.0 cm) with FL cell line  
morphology. It was composed of confluent nests and sheets  
of cells proliferating in no discernable pattern with a  
few groups similar to those described above still  
5 present. Most membrane material apparently had been  
resorbed and was inconspicuous. There was overt invasion  
of host tissue by the FL cell lines with conspicuous bone  
and osteoid production.

Thus, the above results have demonstrated the  
10 viability of encapsulated cells and further that this  
viability could be maintained throughout the implantation  
or injection procedure with the encapsulated cells  
subsequently proliferating within artificial membranes,  
rupturing the membranes and invading into host tissues.

15 Additionally, the above results demonstrate that  
cell lines induced bone production, evidence of the  
maintenance of cell capacity to exhibit their normal  
function following the encapsulation process. Alginate  
and polylysine microcapsules apparently degrade at  
20 different rates, since discernable differences between  
polylysine and alginate encapsulated cells were noted at  
16 days with alginate tending to degrade earlier than  
polylysine.

In order to determine if artificially  
25 encapsulated cells would survive in vivo, 7 nude mice were  
injected with encapsulated cells formulated in varying  
matrices. If injection of encapsulated cells is delayed,  
viability is significantly suppressed. Vital cells  
encapsulated in polylysine and alginate membranes could be  
30 observed 24 days following injection. Surviving cells  
which had been injected alone or in a carrageenan matrix  
were not detectable at 24 days.

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1

## Example 4

In vivo Studies of Treatment of Fracture Non-unions  
Produced in Dogs.

Fracture nonunions were experimentally induced in  
11 research grade dogs. The nonunions were performed by  
surgically removing a 3 mm disc of cortical and cancellous  
bone from the mid-radius. Dogs were then allowed to  
resume normal weight bearing activities, and after 12  
weeks, stable fracture nonunions were produced. The dogs  
were then divided into groups consisting of controls  
receiving only matrix material with no osteoprogenitor  
cells and four animals receiving osteoprogenitor cells  
formatted in varying ways. Each dog received cells which  
had been harvested at the time of the initial surgery and  
maintained in tissue culture as described in Example 1  
above.

In order to facilitate handling during the  
implantation procedures and to insure retention at the  
nonunion site, the encapsulated cells were prepared in a  
gel of low melt agarose (Sigma TYPE VII). A "doughnut"  
prepared with 3 ml of 4% agarose was formed in a 28 mm  
diameter culture dish with a 12 mm diameter post in the  
center. After the agarose had gelled, the centerpost was  
removed. A suspension was prepared from 3 ml encapsulated  
cells and 3ml 2% agarose. The hole in the center of the  
4% agarose doughnut was filled with 1.5 ml of this  
suspension. After the central portion had gelled, the  
entire doughnut was transferred to a cell culture dish,  
covered with DMEM, and returned to the incubator. The  
doughnuts were implanted into the nonunion defects within  
15-18 hours. The outer rim of the doughnut was  
substantial enough to permit gentle handling with  
forceps. The central core was rigid enough to hold the  
encapsulated cells at the implant site, while still  
allowing for diffusion of wound and tissue fluid to the

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1 cells. These discs were then implanted following  
excision of the fibrous nonunion material and the radii  
splinted with a 4-hole stainless steel splint. The dogs  
then resumed weight bearing activity for an additional 12  
5 weeks at which time the animal was sacrificed and material  
taken for detailed histologic evaluation.

The two dogs receiving only polylysine matrix  
material showed a persistent nonunion defect occupying  
approximately 8.5-11% of the original nonunion defect  
10 volume on histomorphometric analysis. The trabecular bone  
volume in these areas was calculated at 6.5 and 24.75%  
respectively with 46.9 and 18.9% fibrous connective tissue  
intermixed as well as a small amount of fibrocartilage.  
In addition, a significant quantity of polylysine matrix,  
15 visible as irregularly shaped refractile material was  
noted throughout the defect. There was a modest  
multinucleate foreign body giant cell response to this  
material as well as minimal chronic inflammatory cell  
infiltration. The histologic features from the two  
20 control dog studies were identical to six control dogs  
from previous studies involving the encapsulation and  
implantation of bone inductive proteins in nonunion  
fractures.

When autologous osteoprogenitor cells were  
25 encapsulated in an artificial matrix of alginate and  
implanted in a dog nonunion, histologic examination showed  
a dramatic and complete healing of the fracture nonunion.  
This was apparent on histomorphometric analysis with 100%  
of the original defect being filled with new bone. The  
30 trabecular bone volume in this area was 55% with no  
interposed fibrous connective tissue. Relatively normal  
cancellous space was present instead. This was in  
dramatic contrast to the controls and other test animals  
receiving inductive proteins. Also apparent were isolated  
35 small cell clusters and groups of cells with round,

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1 elliptically located nuclei and relatively distinct  
cytoplasmic membranes with slight eosinophilia to the  
cytoplasm. These were identical to cell clusters noted in  
the in vitro and nude mouse in vivo experiments. These  
5 cells could be observed within the cancellous space and at  
times in intimate adaptation with an acellular  
eosinophilic homogeneous material consistent with osteoid.

When autologous osteoprogenitor cells were  
encapsulated in a polylysine matrix and implanted in a dog  
10 nonunion, histologic examination 3 weeks demonstrated  
evidence of degrading artificial cell membranes consistent  
with polylysine and a few artificial cell nests as  
described above in the in vitro and in vivo nude mouse  
studies, as well as the dog previously described.

15 Throughout the nonunion site there was evidence of brisk  
osteoblastic activity with production of homogeneous,  
eosinophilic acellular osteoid as noted in the previous  
dog. The histologic features demonstrated healing at a  
significantly advanced stage compared with that  
20 anticipated for control animals from previous nonunion  
experiments. The two remaining dogs each received  
polylysine encapsulated cells or alginate encapsulated  
cells. Both dogs were carried to 13 weeks. The  
polylysine cells showed some evidence of osteoid  
25 production and remnants of artificial cells, but no  
significant fill of the nonunion defect. The same was  
true of the last dog receiving alginate encapsulated cells.

The results of the implantation of  
osteoprogenitor cells encapsulated in alginate (with or  
30 without poly-L-lysine) demonstrated that the method of the  
present invention causes complete healing of the fibrous  
nonunion, the healed fracture being composed of mature  
bone with lamellar characteristics and evidence that  
remodeling of the fracture site into a functional state  
35 had occurred. This Example conclusively demonstrates that

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1 osteoprogenitor cells may be encapsulated in artificial  
membranes, their viability maintained, and these cells  
subsequently implanted in living subjects (mice and  
5 dogs). The cells subsequently proliferate out of the  
artificial confines to produce osteoid and new bone which  
contributes to the healing process.

Although all dogs receiving encapsulated  
osteoprogenitor cells did not demonstrate the same amount  
of nonunion fracture healing, this result may relate to a  
10 number of complex interrelated factors. These include the  
kinetics of artificial cell membrane degradation, cell  
release from artificial membranes, proliferative  
capabilities of individual autologous cells, differences  
inherent in healing capacity of each animal, or  
15 combinations of these.

In addition, bone inductive factors may be  
necessary in the artificial membranes to completely signal  
encapsulated cell populations to begin proliferation  
within the unfavorable environment of a healing wound.  
20 Some of these variables may be overcome by inclusion of  
bone cell differentiation factors within the microcapsule  
at the time of encapsulation.

#### Example 5

##### Device Fixation Enhancement in Primates

25 In order to improve the fixation of titanium bone  
implants, in vivo studies of implantation of  
microencapsules containing osteoprogenitor cells and bone  
inductive factors were performed. Osteoprogenitor cells  
were encapsulated in either alginate or alginate coated  
30 with polylysine as described in Example 1. Additionally,  
microcapsules were prepared as in Example 1 however, bone  
inductive growth factor was also included in the  
microcapsules. The microcapsules were implanted within  
the internal aspects of a titanium bone implant in six  
35 sites in each of two baboons (animal no. 713 and 609) to

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1 determine whether bone growth into titanium prosthetic  
implants could be enhanced, facilitating of the fixation  
of the prothesis in the baboon tibia.

5 One of six titanium implant sites was used as a  
control and received no microcapsule material. Each of of  
the remaining five titanium implant sites in baboon tibia  
recieved the same microencapsulation composition. At one  
week intervals for six weeks, tissue within the internal  
10 aspects of one of the six titanium implant sites in each  
baboon was retrieved for histologic evaluation. The  
status of the encapsulated material and the quantity of  
bone in the internal aspects of the titanium implant was  
determined. Histologic analysis of tissue within the  
15 implant at the site of microcapsule implantation was  
carried out weekly for six weeks. The encapsulating  
materials were highly biocompatible and did not elicit a  
giant cell foreign body response. The amount of  
encapsulating material present in histologic sections  
decreased as the treatment period progressed, indicating  
20 that the implanted microcapsules were biodegraded at the  
site of the titanium prosthetic implant. Gross  
histological examination revealed bone regeneration in all  
titanium prosthetic implant sites which received the  
microcapsule composition of the present invention.  
25 Further analysis may reveal quantitative or qualitative  
differences in the regenerating hard tissue due to the  
presence of the bone inductive growth factor.

The invention now being fully described, it will  
be apparent to one of ordinary skill in the art that many  
30 changes and modifications can be made thereto without  
departing from the spirit or scope of the invention as set  
forth below.

WHAT IS CLAIMED AS NEW AND IS DESIRED TO BE  
35 COVERED UNDER LETTERS PATENT IS:

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## CLAIMS

1. A composition comprising osteoprogenitor cells encapsulated in a biocompatible microcapsule.
2. The composition of claim 1, wherein said microcapsule comprises a biodegradeable polymer.
3. The composition of claim 2, wherein said biodegradeable polymer is selected from the group consisting of alginate, polylysine, methylcellulose, collagen, gellan gum, casein and chitosan.
4. The composition of claim 3 wherein said polymer is alginate.
5. The composition of claim 3 wherein said polymer is polylysine.
6. The composition of claim 1 wherein said microcapsule comprises alginate and polylysine.
7. The composition of claim 1 wherein said microcapsule comprises alginate coated with polylysine.
8. The composition of claim 1 further comprising a material selected from the group consisting of extracellular matrix of chondrocytes (ECM), a hormone, a growth factor, an antiviral agent, and an antibacterial agent.
9. The composition of claim 8 wherein said growth factor is selected from the group consisting of somatomedin, fibroblast growth factor, epidermal growth factor and bone derived growth factor.
10. The composition of claim 1 contained in a hydrogel wafer.
11. The composition of claim 10 wherein said hydrogel wafer comprises a material selected from the group consisting of agar, gelatin, gellan gum and agarose.
12. A method for promoting bone regeneration, comprising administration of the composition of any one of claims 1 - 11, inclusive, to an individual in need of said treatment.

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1           13.    The method of claim 12 wherein said individual is a mammal.

          14.    The method of claim 13 wherein said mammal is selected from the group consisting of a human, a dog, a  
5   cat, and a horse.

          15.    The method of claim 14 where in said mammal is a human.

          16.    A composition comprising a material resistant to degradation in biological fluids, wherein  
10   said resistant material is selected from the group consisting of titanium oxide, hydroxyapatite, biocompatible metal compositions and biocompatible ceramic compositions; hard tissue promoting factors bound to the resistant material; and contained within a readily  
15   degradeable polymer.

          17.    The composition of claim 16 additionally comprising unbound hard tissue promoting factors.

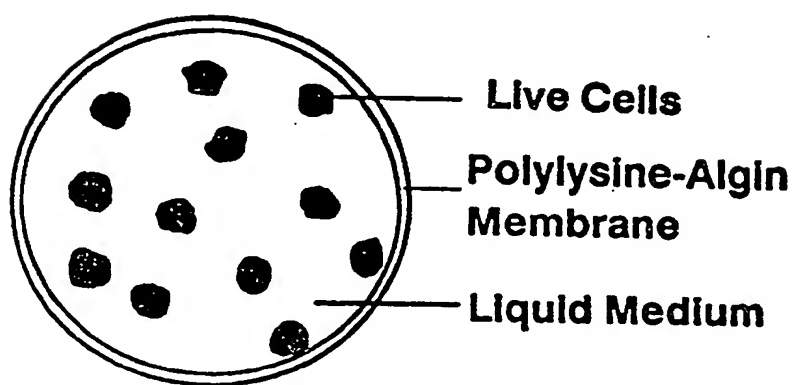
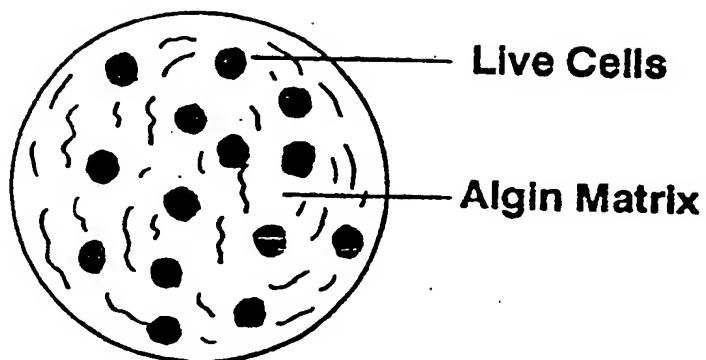
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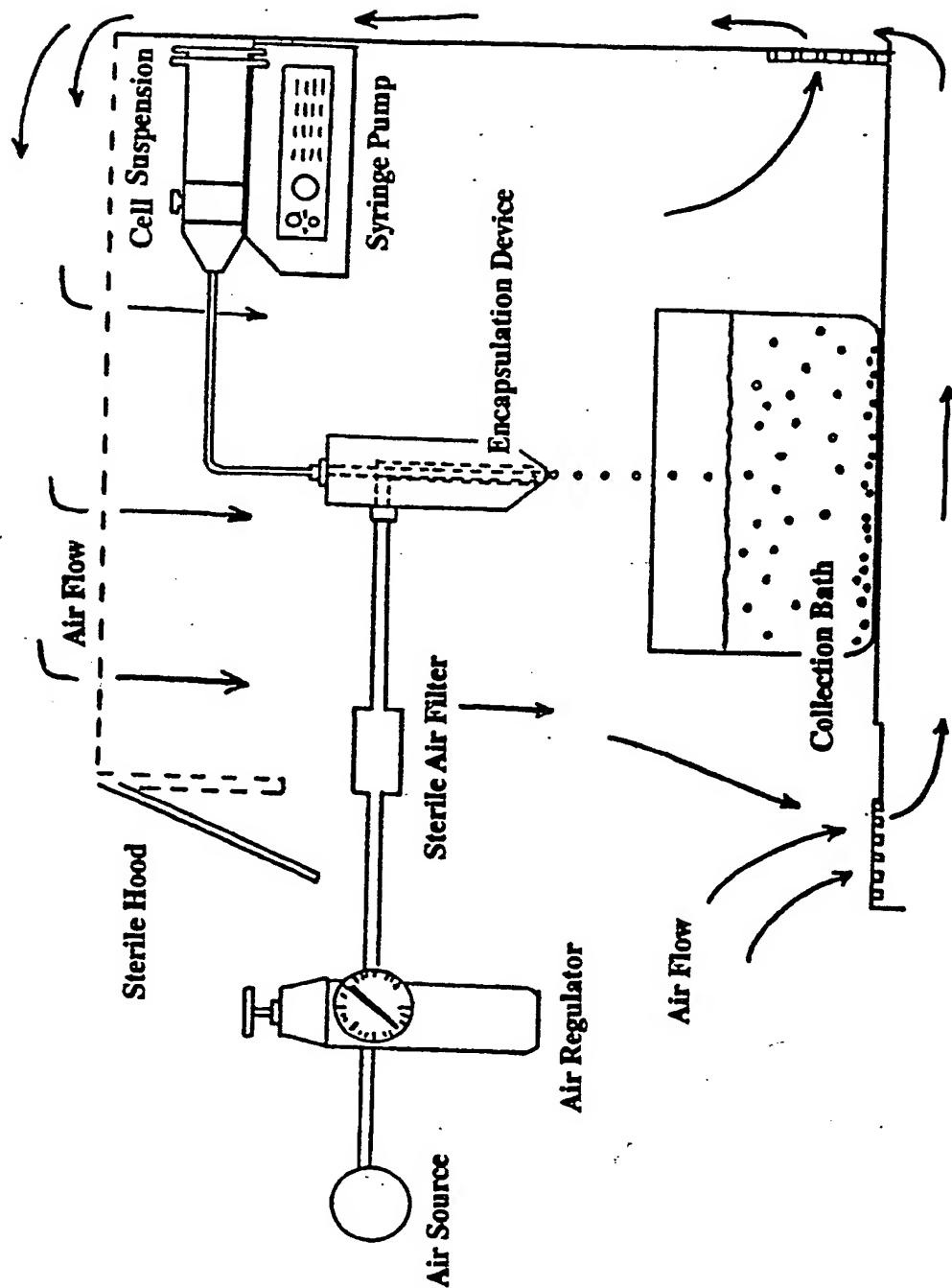
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## TWO TYPES OF ARTIFICIAL CELLS

Figure 1

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# CELL ENCAPSULATION PROCESS

Figure 2

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US90/04381**

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC  
**IPC (5): A61K 9/14, 9/16, 9/52, 9/64, 37/24; C12N 11/04, 11/10, 11/12**  
**U.S. CL. 424/484, 485, 488, 491,; 435/178, 182; 530/399**

## II. FIELDS SEARCHED

### Minimum Documentation Searched <sup>7</sup>

Classification System	Classification Symbols
U.S.	424/484, 485, 488, 491; 435/178, 179, 182 530/399

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>

Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A, P	US, A, 4,904,259 (ITAY) 27 FEBRUARY 1990 See Abstract.	1, 2, 12-15
Y	US, A, 4,642,120 (NEVO) 10 FEBRUARY 1987 See Abstract and column 4, lines 35-36.	1, 2, 8, 9, 12-15
Y	US, A, 4,609,551 (CAPLAN) 02 SEPTEMBER 1986 See Abstract, column 2, lines 1-2, 17-22, 54 and column 3, lines 34-48.	1-3, 8, 12-15
Y	US, A, 4,647,536 (MOSBACH) 03 MARCH 1987 See Abstract and column 1, lines 10-15.	1, 2, 3
Y	US, A, 4,663,286 (TSANG) 05 MAY 1987 See Abstract, column 1, lines 5-7 and column 3, lines 4-38.	1-7
Y	US, A, 4,391,909 (LIM) 05 JULY 1983 See Abstract, column 4, lines 9-16, column 9, and Example 1.	1-7
Y	US, A, 4,673,566 (GOOSEN) 16 JUNE 1987 See Abstract, column 8 and Example 1.	1-7, 10, 11

### \* Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATE

Date of the Actual Completion of the International Search <b>02 OCTOBER 1990</b>	Date of Mailing of this International Search Report <b>09 JAN 1991</b>
International Searching Authority <b>ISA/US</b>	Signature of Authorized Officer <i>S. Kishore</i> <b>Gollamudi S. Kishore</b>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4,798,786 (TICE) 17 JANUARY 1989 See Abstract, column 10 and claim 3.	1-3, 11-15
Y	US, A, 4,620,327 (CAPLAN) 04 NOVEMBER 1986 See Abstract, column 2, lines 4-54 and column 3, lines 27-34.	16 & 17
Y	US, A, 4,610,692 (EITENMULLER) 09 SEPTEMBER 1986; See Abstract.	16 & 17
Y	US, A, 4,595,713 (ST. JOHN) 17 JUNE 1986 See Abstract, and column 6, lines 23-41.	16 & 17
Y	US, A, 4,888,366 (CHU) 19 DECEMBER 1989 See Abstract, column 2, lines 54-64 and column 7, lines 7-65.	16 & 17

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup>not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-15: A composition comprising osteoprogenitor cells encapsulated in a microcapsule.
- II. Claims 16-17: A composition containing no osteoprogenitor cells.  
(See attachment)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
  
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Con't. from PCT/ISA/210 supplemental sheet (2).

The international application lacks unity of invention under PCT Rule 13 because of the following reason:

Inventions I and II are independent and distinct in that the composition in invention I contains osteoprogenitor cells where as the composition in invention II does not require the presence of these cells, but contain additional factors.